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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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|---|---|---|---|
| Applicant: | Stace Lindsay et al. | Art Unit: | 1632 |
| Serial No.: | 10/030,351 | Examiner: | Valarie E. Bertoglio |
| Filed: | June 7, 2002 | Customer No.: | 21559 |
| Title: | EXPRESSION OF SECRETED HUMAN ALPHA-FETOPROTEIN IN TRANSGENIC ANIMALS | | |

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Arlington, VA 22313-1450

DECLARATION OF EDWARD J. STEWART
UNDER 37 C.F.R. § 1.132

I, Edward J. Stewart, declare:

1. I am the Director of Business Development for Merrimack Pharmaceuticals, Inc. ("Merrimack"), the licensee of United States Patent Application Serial No. 10/030,351, which was filed on June 7, 2002.

2. I am responsible for the clinical development of products containing recombinant human alpha-fetoprotein (rHuAFP) at Merrimack. I hold an MBA from the Johnson Graduate School of Management at Cornell University. I have been employed at Merrimack since 2001.

3. I have read and understood the Office Action, dated March 22, 2005. This Declaration is presented to overcome the rejection of claims 1, 3, 5-7, 10, and 11 under 35 U.S.C. § 112, first paragraph, for lack of enablement.

4. I have interacted with the inventors, and the scientists working under their direction, who have confirmed that the methods of the invention have been used successfully and reliably to generate transgenic goats capable of expressing and secreting rHuAFP in their milk.

5. The materials and methods employed were known to those skilled in the art of transgenic animal production at the time the application was filed or are provided by the instant specification and were as follows:

**Materials**

Restriction enzymes were obtained from New England Biolabs, Beverly, MA. Nylon membranes (Immobilon P) for Western blotting were obtained from Owl Separation Systems Portsmouth, NH. Oligonucleotides were obtained from Operon Technologies, Inc., Alameda, CA. Sequence analysis was performed by Sequegen Company, Worcester, MA.

Recombinant DNA libraries were obtained from Stratagene (La Jolla, CA; λFix, human genomic placenta) or Clontech, Palo Alto, CA; human fetal liver cDNA).

Rabbit anti-alpha-fetoprotein antibody was obtained from Caltag Laboratories Burlingame,CA. Goat anti-rabbit IgG coupled to horse-radish peroxidase came from ICN (Aurora, OH) and alpha-fetoprotein purified from human cord serum is from Lee Scientific, Inc., St. Louis, MO.

**Methods**

*Recombinant DNA Procedures*

Recombinant DNA procedures were performed following Sambrook, Fritsch, and Maniatis. Genomic and cDNA libraries were screened with radiolabeled oligonucleotide probes derived from coding exons at the beginning (5'), middle and end (3') of the human AFP gene (GenBank Accession #M16110).

*Preparation of DNA for Microinjection and Transfection*

Transgene DNA was separated from the vector backbone by digesting the plasmid to completion with SalI and NotI. Digests were then electrophoresed in an agarose gel, using 1X TAE (Maniatis et. al., 1982) as running buffer. The region of the gel containing the DNA fragment corresponding to the expression cassette was visualized under UV light (long wave).

The band containing the DNA of interest was excised, and the DNA isolated by electro-elution in 1X TAE. This procedure was applied for each expression cassette.

Following electro-elution, DNA fragments were concentrated and further purified by using the "Wizard DNA Clean-Up System" (Promega, Cat # A7280), following the manufacturers protocol. The final elution was performed using 125 µl of microinjection buffer (10 mM Tris pH 7.5, EDTA 0.2 mM). The concentration of this stock solution was evaluated by comparative agarose gel electrophoresis with standards of known concentration. The stock solution aliquots for microinjection were diluted in microinjection buffer just prior to microinjections so that the final concentration of each fragment was 0.5 ng/ml.

*Mouse Microinjections*

CD1 female mice were superovulated and fertilized ova were retrieved from the oviduct. The male pronuclei were then microinjected with DNA diluted in microinjection buffer. Microinjected embryos were either cultured overnight in CZB media or transferred immediately into the oviduct of pseudo-pregnant recipient CD1 female mice. Twenty to thirty 2-cell or forty to fifty 1-cell embryos were transferred to each recipient female and allowed to proceed to term.

*Cell Line Isolation*

Goat fetal fibroblast cells were isolated from goat fetal tissue from pregnant goats. The rHuAFP transgene and neomycin resistance gene were prepared, and co-transfected into the goat fetal fibroblasts using LipofectAmine at 1-2 µg of transgene DNA fragments/$10^6$ cells. Colonies of neomycin-resistant cells were isolated following G418 selection. Isolated clones were expanded and selected cell lines were cryo-preserved. These cell lines were subjected to PCR analysis using rHuAFP specific primers to determine the presence of the transgene. In addition, FISH analysis of the cell lines was carried out to confirm the integration of the transgenes.

*Embryo Collection, Nuclear Transfer, and Embryo Transfer*

For nuclear transfer, somatic cells were isolated from either fetal tissues or skin biopsies, and transfected characterization, as is described above. The transfected and characterized somatic cells containing the hAFP transgene were placed into culture and prepared for nuclear transfer.

Oocytes collected surgically were enucleated through mechanical removal of the metaphase plate of the MII oocyte. These enucleated oocytes (cytoplast) where then reconstructed with an individually isolated transfected somatic cell (karyoplast). Once reconstructed, the couplet (enucleated oocyte and somatic cell) was fused together by an electrical pulse which simultaneously activated the reconstructed embryo. The activated embryo was then placed into culture, maintained for 24-48 hours, and assessed for embryo viability and development prior to embryo transfer.

Following the nuclear transfer process and embryo culture, viable and developing embryos were transferred to suitable recipient animals. Approximately 3-10 embryos were surgically transferred to recipient animals by depositing them into the oviduct, ipsilateral to a CL, in a small volume of media by precise cannulation of the oviduct with a glass pipette.

*Identification of Founder Animals*

Genomic DNA was isolated from goat ear tissue and white blood cells. The isolated DNA was extracted successively with saturated phenol, phenol:isoamyl alcohol, and chloroform prior to precipitation with 6M NaCl and ethanol and analyzed by polymerase chain reaction (PCR) to detect the β-casein-alpha-fetoprotein junction DNA sequences present only in the transgenes. For the PCR reactions, approximately 250 ng of genomic DNA was diluted in 50 µl of PCR buffer (20 mM Tris pH 8.3, 50 mM KCl and 1.5 mM $MgCl_2$, 100 mM deoxynucleotide triphosphates, and each primer at a concentration of 600 nM) with 1.0 unit of Taq polymerase and in a MJ Research DNA Engine using the following cycling program:

Cycling Program:

| | | |
|---|---|---|
| 1 cycle | 94°C | 60 sec |
| 5 cycles | 94°C | 15 sec |
| | 55°C | 15 sec |
| | 74°C | 30 sec |
| 30 cycles | 94°C | 15 sec |
| | 55°C | 15 sec |
| | 74°C | 30 sec |
| 1 cycle | 74°C | 10 min |

Primers used: Oligo GTC17 GATTGACAAGTAATACGCTGTTTCCTC
Oligo AFP-PCR3 TTTGTAAACCTCTTGTAAAGTTACAAG

Oligo GEX7F CCAGGCACAGTCTCTAGTCTA
Oligo GEX7R GGACAGGACCAAGTACAGGCT

*Southern Blot Analysis*

Five micrograms of genomic DNA were digested with 100 units EcoRI as described by Maniatis followed by electrophoresis through a 0.8% agarose gel. The gel was then blotted to a charged Nylon membrane (Genescreen Plus, New England Nuclear) by capillary action in 0.4N sodium hydroxide and UV crosslinked (Stratalinker, Sratagene). After prehybridization in hybridization buffer (5X SSC, 50% formamide, 10% dextran sulfate, 20 mM sodium phosphate, 1X Denhardt's, 0.5% SDS) containing 20 μg/ml denatured herring sperm DNA, probe was added and the blot incubated overnight at 42° C. Blots were washed as follows and autoradiographed:

1X SSC, 1% SDS at room temperature x 20 min.
0.5X SSC, 0.5X SDS at room temperature x 20 min.
(0.1X SSC, 0.1% SDS at 65° C x 20 min.) x 3

*Western Blotting*

The immunoblotting procedures of Harlow and Lane were used for the immuno-detection of proteins. Milk samples were diluted 1:20 in PBS, then mixed 1:1 with 2X SDS gel loading buffer (50 mM Tris HCl, pH 6.8, 2 % SDS, 10 % glycerol, 10% β-mercaptoethanol), heated at 65°C for two minutes, and subjected to SDS PAGE. The proteins in the gel were transferred onto Immobilon P membranes in transfer buffer (50 mM Tris, 380 mM glycine, 0.1 % SDS, 20 % methanol) by electroblotting. For immunostaining, the membrane was incubated with a blocking buffer (4% Non-fat Dry Milk, BioRad, Hercules, CA, in PBS containing 0.01%Tween-20) at room temperature (RT) for 1 hour. Membranes were then incubated with the anti-hAFP antibody (1:5000 in blocking buffer) forl hour at RT. Following three short washes in $dH_2O$, membranes were incubated with the secondary antibody (1:10000 in blocking buffer) for 1 hour at RT. Membranes were then washed 3X $dH_2O$ for 4 min. each, then PBS/Tween-20 and again for 6X in $dH_2O$ before development with the chemiluminescent substrate (ECL) followed by autofluorography.

*Fluorescence In Situ Hybridization (FISH) Analysis*

Standard culture and preparation procedures were used to obtain metaphase and interphase nuclei from cultured blood lymphocytes from founder goat F093.

Nuclei were deposited onto slides and were hybridized with a digoxigenin-labeled probe derived from a construct containing 8kb of the genomic sequence for human AFP. Bound probe was amplified using a horseradish peroxidase-conjugated antibody and detected with tyramide-conjugated fluorescein isothiocyanate (FITC, green fluorochrome). Nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI, blue dye). FISH images were obtained using MetaMorph software.

6. The methods described in paragraph 5 produced the following results:

*Analysis of Transgenic Mice Derived from Genomic DNA Constructs*

Transgenic female mice containing the rHuAFP transgene were identified by PCR and are listed in Table I. Milk from these animals was analyzed by Western blot and expression levels were estimated by comparison to hAFP standards of known concentration. Results of this expression analysis are shown in Table I.

Table 1. Mouse Milk Expression Results

| 1st Generation Mouse ID # | 2nd Generation Mouse ID # | Expression Level (mg/ml) |
|---|---|---|
| 9F | | 1 |
| 10F | | 10 |
| | 109 | 2 |
| | 110 | 2 |
| | 111 | 1 |
| | 112 | 1 |
| 37F | | 10-20 |
| | 116 | 2 |
| | 118 | 5 |
| 44 | | <1 |
| | 120 | 4 |
| | 121 | 4 |
| 74F | | 2 |
| | 129 | 1 |
| 85F | | 1 |

*Genetic Analysis of Founder Goat F093*

A healthy female goat (F093) was born March 11, 2002 (see photograph of "Merri" enclosed as Exhibit A). To determine if this goat carries the rHuAFP transgene, PCR analysis of blood and ear tissue was performed (Exhibit B). Initially, two PCR primer pairs were used at the same time. The first pair is specific to the transgene and the resultant 332 bp product spans the junction of the 5' beta-casein and the 5' rHuAFP sequences. The second primer pair recognizes goat beta-casein exon seven which is not present in the transgene construct and gives a product of 439 bp. Exhibit C shows that the rHuAFP transgene is present in goat F093, in both the blood and ear samples. A transfected cell line that had been characterized earlier was used as a positive control. Ear tissue from an abortus that had been shown to carry the rHuAFP transgene was also used as a positive control.

After confirming the genotype, Southern Blot Analysis was performed to estimate copy number and rule out gross transgene rearrangements. The DNA probe used was a XhoI/HindIII fragment of the 3' beta-casein gene found in the transgene and the endogenous goat beta-casein gene. By comparing the relative intensities of the transgene and the endogenous gene, one can estimate the transgene copy number. The endogenous gene signal represents two copies of the gene in a diploid genome. As can be seen in Exhibit C, the two bands in the F093 sample appear quite similar in intensity. Scanning densitometry (Molecular Dynamics) confirms a one-to-one ratio (the F026 abortus has a 13:1 ratio by densitometry).

FISH images of metaphase chromosomes and interphase nuclei showing the rHuAFP transgene are shown in Exhibit D. The transgene signal is located towards the "q" terminal end on a mid to large sized autosomal chromosome. FISH analysis is consistent with the existence of a single transgene integration site.

7. The data described in paragraph 6 clearly demonstrate that transgenic mice and a founder transgenic goat were successfully generated according to the methods disclosed in the present specification. The data confirm that rHuAFP was cloned and expressed in the milk of several lines of transgenic mice as a genomic "mini-gene." The expression of rHuAFP is under the control of the goat β-casein regulatory elements in the mice and goat. Expression levels in non-mosaic mice (mice able to pass the transgene on to subsequent generations) ranged from 1.0 to 20 mg/ml. The transgene products from all constructs were immunoreactive with a hAFP-specific antibody.

The data also confirm that rHuAFP was cloned and expressed in the milk of a founder transgenic goat bearing the same genomic transgene used to express high levels of rHuAFP in mice. Genetic analysis of this goat, F093, confirms that she is indeed transgenic and harbors approximately two copies of the transgene in a single integration site. At this time, seventeen transgenic goats have been successfully and reliably produced using the methods disclosed in the present specification or known in the art at the time of filing.

8. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Respectfully submitted,

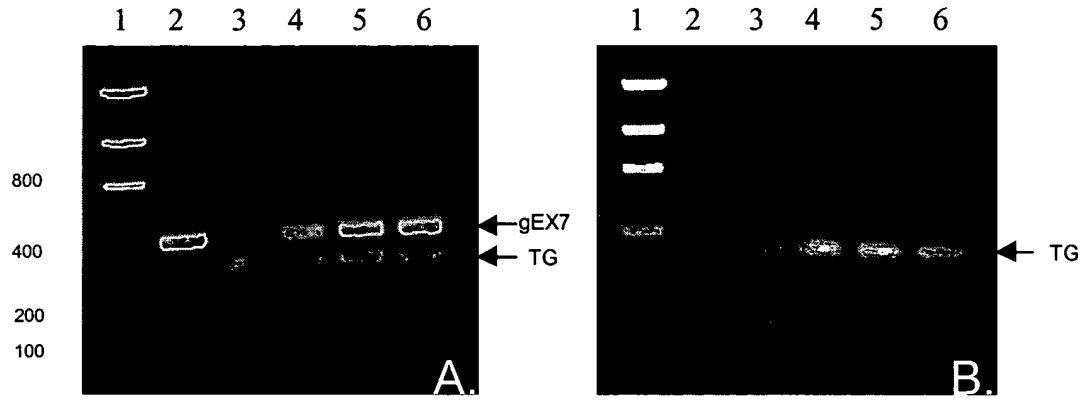
Date: _____

_____

Edward J. Stewart
Director of Business Development
Merrimack Pharmaceuticals, Inc.

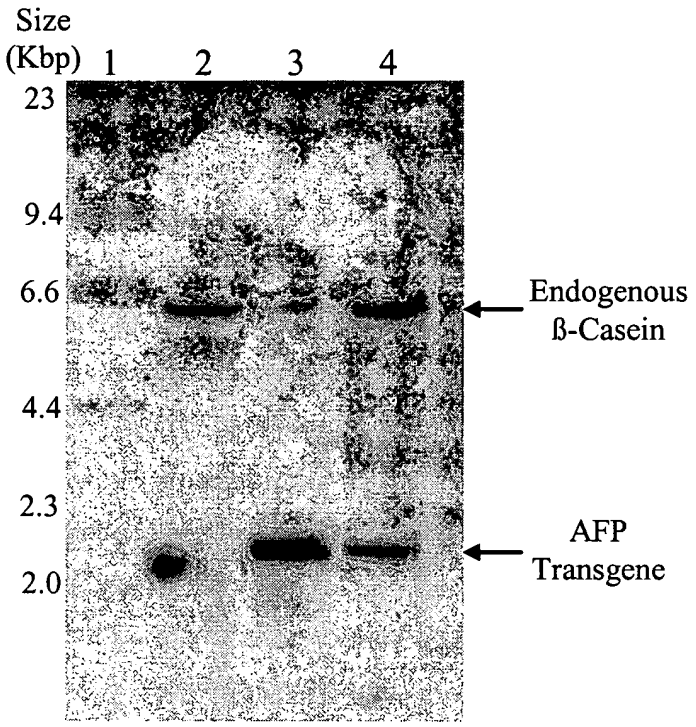
# Exhibit A

# Exhibit B



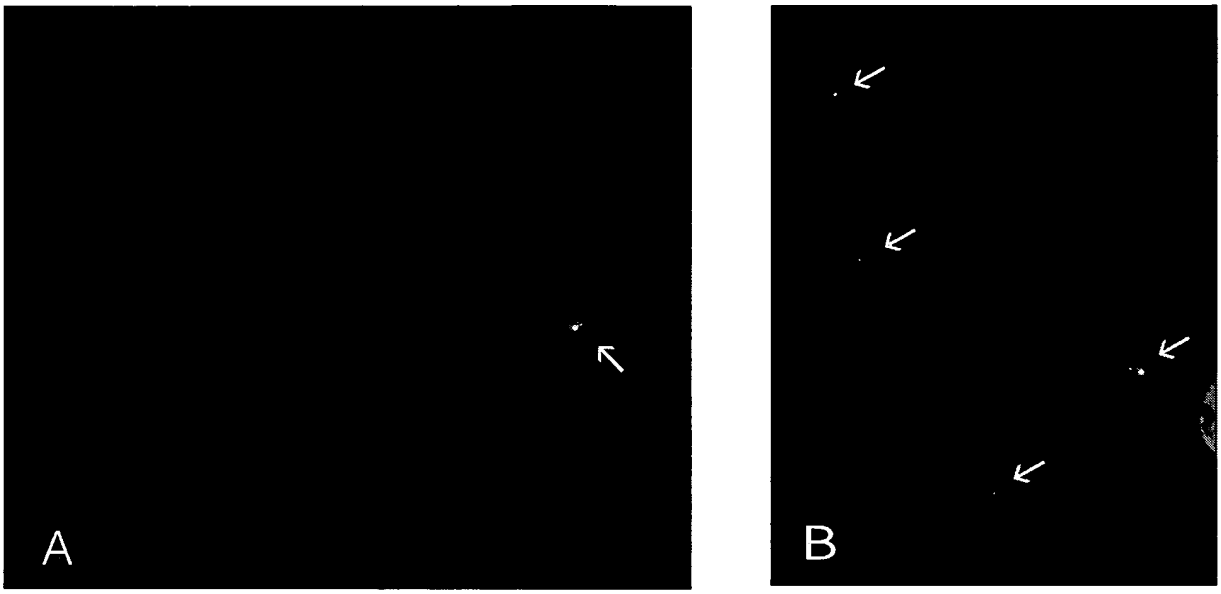

*PCR Analysis of Founder Goat, F093, Blood and Ear Tissue*
Panel A: Duplex PCR analysis using the goat exon 7 primer pair (410 bp product) with a rHuAFP-specific primer pair (332 bp product). Panel B: rHuAFP-specific primer pair alone. The template DNA in both experiments was the same. Lane 1: DNA size standards. Lane 2: Non-transgenic goat blood sample. Lane 3: rHuAFP positive goat cell line (clone 7). Lane 4: Ear tissue from rHuAFP-positive abortus, F026. Lane 5: Blood tissue from F093. Lane 6: Ear tissue from F093.

# Exhibit C




*Southern Blot Analysis of Founder Goat, F093*
5 mg DNA was digested with EcoRI, separated by electrophoresis, and blotted to Genescreen Plus. Blot was then hybridized with a goat beta-casein probe, washed and autoradiographed. Lane 1: lHindIII,molecular weight markers. Lane 2: Non-transgenic goat ear tissue DNA; Lane 3: F026, rHuAFP-positive abortus ear tissue DNA. Lane 4: F093, founder goat blood DNA.

# Exhibit D




*FISH Analysis of Founder Goat, F093*
Panel A: Representative example of metaphase chromosomes of F093 cultured leukocytes. The transgene signal is green and is indicated by the arrow. The chromosomes are visualized with the blue DAPI stain. Magnification: 1000X. Panel B: Representative field of interphase nuclei of F093 cultured leukocytes. The transgene signals are green and indicated by the arrows. The DNA in the nuclei is visualized with the blue DAPI stain. Magnification: 1000X.